(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
11 December 2003 (11.12.2003)

PCT

(10) International Publication Number
WO 2003/102239 A3

(51) International Patent Classification[7]: C12Q 1/68

(21) International Application Number: PCT/IN2003/000204

(22) International Filing Date: 30 May 2003 (30.05.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
487/MUM/2002 31 May 2002 (31.05.2002) IN

(71) Applicant *(for all designated States except US)*: **SECRETARY, DEPARTMENT OF ATOMIC ENERGY** [IN/IN]; Government of India, Anushakthi Bhavan, Chatrapathi Shivaji Maharaj Marg, Mumbai 400001 (IN).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **ISLAM, Amirul** [IN/IN]; Board of Radiation and Isotope Technology, Department of Atomic Energy, CCMB Campus, Hyderabad 500007 (IN). **HAZRA, Papia** [IN/IN]; Board of Radiation and Isotope Technology, Department of Atomic Energy, CCMB Campus, Hyderabad 500007 (IN).

(74) Agents: **MAJUMDAR, S.** et al.; S. Majumdar & Co., 5, Harish Mukherjee Road, Kolkata 700025 (IN).

(81) Designated States *(national)*: CN, JP, US.

(84) Designated States *(regional)*: European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR).

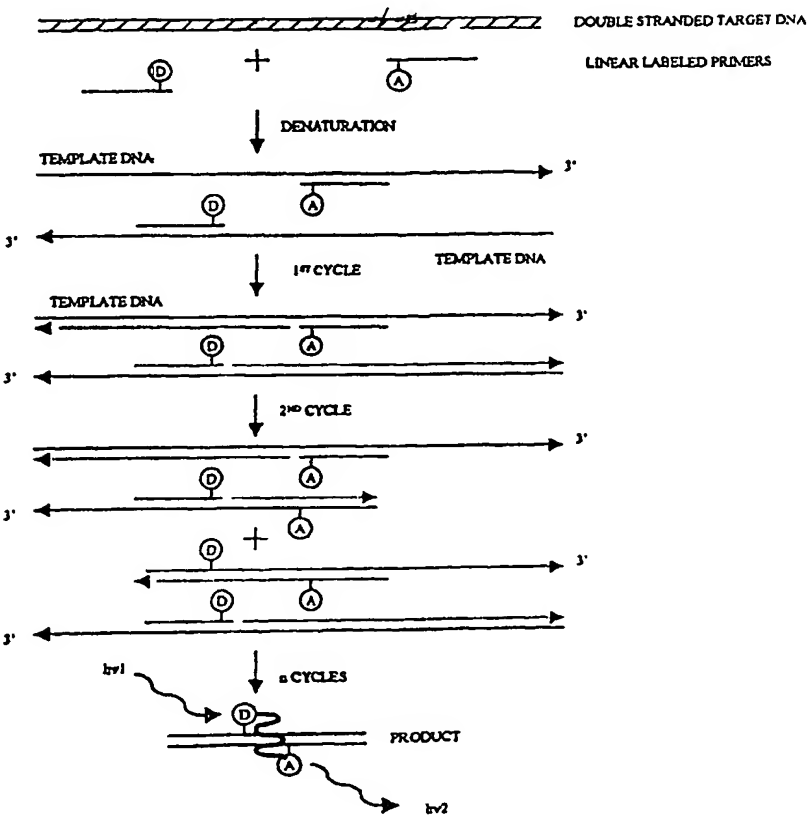
Published:
— *with international search report*
— *with amended claims and statement*

(88) Date of publication of the international search report: 27 May 2004

Date of publication of the amended claims and statement: 15 July 2004

[Continued on next page]

(54) Title: MET/FRET BASED METHOD OF TARGET NUCLEIC ACID DETECTION WHEREBY THE DONOR/ACCEPTOR MOIETIES ARE ON COMPLEMENTARY STRANDS



(57) Abstract: Disclosure of a method for the detection and quantitation of polynucleotide sequences in a sample of biological or non-biological material through target poly nucleotide sequence amplification whereby MET/FRET occurs between a donor moiety and an acceptor moiety provided separately on at least two separate oligonucleotides that are part of the opposite complementary strands of a nucleic acid segment.

WO 2003/102239 A3

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

AMENDED CLAIMS

[received by the International Bureau on 27 May 2004 (27.05.04);
Original claims 1-62 replaced by new claims 1-61 (25 pages).]

1. A method of detection and/or quantification of target nucleic acid sequence and/or by nucleic acid amplification reaction comprising:

MET/FRET between a donor moiety and an acceptor moiety provided separately on at least two separate oligonucleotides that are part of the opposite complementary strands of a nucleic acid segment such that the 3' ends of the two above said oligonucleotides remain separated from each other by 0-20 / 25 nucleotide pairs in the final amplification product and the donor and acceptor moieties, which otherwise remain separated from each other in absence of target amplification, come close to each other within 0-20/25 nucleotide pairs in the final amplification product.

2. A method of direct monitoring of a specific amplification product in an amplification reaction of a nucleic acid target comprising MET/FRET between a donor moiety and an acceptor moiety provided separately on at least two separate oligonucleotides that are part of the opposite complementary strands of a nucleic acid segment with the donor and acceptor moieties, which otherwise remain separated in absence of target amplification, coming close to each other remaining separated from each other by 0-25 nucleotide pairs in the final amplification product such that signal is generated as a result of bringing the two otherwise separated MET/FRET moieties close together in the amplified product resulting in energy transfer and measured by exciting the donor moiety and measuring the acceptor emission and/or donor emission.

3. A method as claimed in anyone of claims 1 or 2 wherein the two labeled oligonucleotides are selected from two amplification primers, one amplification primer and the other probe.

4. A method as claimed in anyone of claims 1 to 3 wherein amplification product of the size close to that of a primer dimer is used , wherein the 3' ends of the two amplification primers getting incorporated on two opposite strands and remain

separated from one another by 0-25 nucleotide pairs in the final amplification product resulting in improved signal to noise ratio due to improved yield of amplification .

5. A method as claimed in anyone of claims 1 to 4 wherein atleast the acceptor moiety is provided quenched in the unincorporated or non-hybrdized form to reduce the background in emission wave length region of the acceptor thus resulting in improved signal to noise ratio.

6. A method of detection of target nucleic acid sequence as claimed in claim 1 comprising (i) providing at least two oligonucleotides as a pair of primers for amplification of said target sequence; (ii) subject the target sequence to amplification such that the 3' ends of said pair of primers are on two opposite strands and separated from one another by 0-25 nucleotide pairs in the final amplification product; and (iii) carrying out denaturation step and at least a selective annealing step in each cycle.

7. A method of claim 6 wherein said denaturation carried out is less than 20 seconds preferably less than 10 seconds, said annealing carried out is less than 15 seconds preferably less than 5 seconds and extension of less than 10 seconds preferably 0 seconds in each cycle for high through put PCR or nucleic acid target analysis

8. A method of anyone of claims 1 to 7 wherein a first oligonucleotide is labeled with a donor MET moiety at or near preferably near 3' end preferably within 2-10 nucleotides away from 3' end and the second oligonucleotide is labeled with an acceptor MET moiety, at or near preferably near 3' end within 2-10 nucleotides away from 3' end, the said donor and acceptor MET moieties belonging to a molecular energy transfer pair and so configured that the donor and the acceptor moieties come within MET distance, 10–80 Angstrom or the nucleotides to which the MET moieties are attached come close remaining separated by 2–20 nucleotides preferably by 4 – 12 nucleotides in the amplified product at the time of signal measurement.

9. A method of anyone of claims 1 to 8 wherein the labeled oligonucleotides are selected from (a) linear, (b) hair-pin or (c) otherwise configuration.

10. A method as claimed in any one of claim 1-9 wherein the oligonucleotide labeled with the acceptor moiety or both the oligonucleotides labeled separately with donor or acceptor moiety are provided in quenched condition with quencher or quenchers or by providing them with hair-pin stem structure such that the emission energy of the acceptor or both the acceptor and the donor remain quenched when not incorporated into the amplification product thus reducing the background emission in the emission wave length region of the acceptor.

11. A method as claimed in claim 10 wherein said quenching is achieved following anyone of:

(I) at least the oligonucleotide labeled with the acceptor provided in a hair-pin quenched configuration, where the acceptor is provided quenched with a quencher or both the donor as well as the acceptor labeled oligonucleotides are provided in hair-pin quenched configuration so that both the donor and the acceptor moieties are provided quenched with two separate quenchers, the quenchers are provided on the same oligonucleotides and attached at or near the respective 5' ends, the quencher and the acceptor or the donor are on two opposite strands of the stem structure of the hair-pin and part of the same oligonucleotide. In the event of formation of hair–pin stem structure the nucleotide to which the donor or the acceptor moiety is attached is complementary and opposite to the nucleotide to which the quencher is attached or the nucleotide to which the donor or the acceptor moiety is attached and the complement of the nucleotide to which the quencher is attached are within five nucleotides, the donor labeled and or acceptor labeled hair-pin quenched oligonucleotides remain quenched when not incorporated into the amplification product.

(II) using additional one or two oligonucleotides as the case may be each being labeled separately at or near 5' end with suitable quencher for the acceptor or the donor MET moiety such that one member of the quencher labeled additional oligonucleotide is fully or partly complementary to the acceptor labeled oligonucleotide as long as the complementarity is maintained resulting in quenching

of the acceptor when the acceptor labeled oligonucleotide is not incorporated into the amplification product and the second member of the quencher labeled additional oligonucleotide is fully or partly complementary to the donor labeled oligonucleotide as long as the complementarity is maintained resulting in quenching of the donor when the donor labeled oligonucleotide is not incorporated into the amplification product ; and

(III) by providing the acceptor labeled oligonucleotide linked to another suitable oligonucleotide complementary partly or fully to this acceptor labeled oligonucleotide and labeled with a quencher at or near its 5' end through a non-nucleotide organic linker or linker and spacer or by providing both the acceptor and donor labeled oligonucleotides linked to two separate additional suitable oligonucleotides fully or partly complementary to the acceptor and donor labeled oligonucleotides respectively through non-nucleotide organic linkers or linkers and spacers and labeled at or near their 5' ends with two quenchers respectively so that the quenchers can quench the acceptor and the donor when the acceptor and the donor labeled oligonucleotides are not incorporated into the amplification product.

12. A method of anyone of claims 1 to 11 wherein a first oligonucleotide primer pair selected to amplify a first segment of the target nucleic acid is used at appropriate concentrations, a second oligonuceotide primer pair selected to amplify a second segment of the first segment at appropriate concentrations used in nested PCR when the second oligonucleotide primer pair is any of the labeled oligonucleotide primer pairs of claims 1-11.

13. A method of anyone of claim 1-12 wherein a first oligonucleotide primer pair selected to amplify a first segment of the target nucleic acid is used at appropriate concentration, where one of the said oligonucleotide primer pair is a first member of the labeled primer pairs of any of the claims 1-12, a third polymerase extendable oligonucleotide suitably labeled for MET, is the second member of the labeled primer pairs of any of the claims 1-11 and designed to amplify a second segment of the first segment in association with the above first member is nested and signal being generated

on said selective nested amplification of the target nucleic acid by bringing the donor and the acceptor moieties close together within MET/FRET distance 0-25 nucleotide pairs preferably between 4-10/15 nucleotide pairs.

14. A method as claimed in claim 1-13 , wherein said nucleic acid amplification reaction comprise any known nucleic acid amplification reactions preferably polymerase chain reaction comprising the steps of adding a polymerase, reaction buffer, deoxy nucleoside triphosphates in addition to the effective amounts of amplification primers to the sample, cycling the sample, between at least a denaturation temperature and an annealing temperature, exciting the reaction mixture with the donor exciting radiation or light, measuring the increase in emission of the acceptor MET moiety, optionally reduction in donor emission.

15. A method as claimed in claim 13, wherein said step of amplifying the target sequence comprise a nucleic acid amplification reaction carried out using one labeled oligonucleotide as one of the two amplification primers of the target sequence amplification reaction along with the other unlabeled primer and a third labeled oligonucleotide, which is not extendable by polymerase, the said labeled oligonucleotide primer being labeled at or near 3'end with a donor or an acceptor MET moiety of a donor-acceptor MET pair and the said third oligonucleotide being labeled at or near 3' end respectively with an acceptor or donor MET moiety of the above MET pair such that upon successful amplification of the target sequence the labeled primer gets incorporated into one of the two strands of the amplification product and the third labeled oligonucleotide hybridizes to this strand of the amplification product into which the labeled oligonucleotide primer get incorporated thus bringing the donor and the acceptor MET moieties within a MET distance 0-20 nucleotides of one another preferably between 1-10/15 resulting in MET between the two moieties; the above said amplification reaction comprising the steps of adding polymerase, reaction buffer, deoxy nucleoside triphosphates in addition to the effective amounts of the amplification primers to the samples, cycling the sample between at least a denaturation temperature and an elongation temperature, exciting the reaction mixture with the donor exciting radiation or light, measuring the emission of the acceptor MET moiety and optionally

the reduction in donor emission, thus allowing detection of nucleic acid target without creating inhibition to amplification reaction and signal measurement without loss of signal; further the 5' end of the third oligonucleotide can be suitably modified to avoid it's degradation.

16. A method as claimed in any one of claims 12 to 15 wherein

(a) atleast the oligonucleotide labeled with the acceptor is provided in quenched configuration such that the acceptor remains quenched when the acceptor labeled oligonucleotide is not incorporated into or not hybridized to the amplification product, thus reducing the background and remains unquenched in separated or open configuration of the oligonucleotide allowing MET between donor and acceptor and signal generation when incorporated into or hybridized to the amplification product,

(b) the amplified sample is illuminated with light absorbed by the donor MET moiety, and

(c) monitoring the sensitized emission from the acceptor and optionally reduction in emission from donor of the MET pair moieties.

17. A method as claimed in anyone of claims 1 to 16 wherein a first oligonucleotide of linear or hair-pin configuration labeled with a donor moiety at or near preferably near its 3' end and a second oligonucleotide singly labeled at or near its 3' end with an acceptor moiety capable of absorbing the energy or light emitted by the donor, where the acceptor is selected from a fluorophore or a quencher preferably a quencher including DABCYL or its analogue or nanogold particle black hole quencher, the donor moiety of the first oligonucleotide kept quenched when the first oligonucleotide is not incorporated into the amplification product either by providing a third oligonucleotide fully or partly complementary to the first oligonucleotide seperately or linked to first oligonucleotide through an organic non-nucleotide linker and labeled at or near its 5' end with a quencher moiety or by providing the first donor labelled oligonucleotide as hair-pin oligonucleotide with a quencher at or near its 5' ends so configured that the quencher comes in close proximity to the donor moiety in its stem structure, the quencher is selected to be capable of absorbing the energy or light emitted by the donor,

and selected to be a fluorophore or a non-radiative quencher preferably a quencher including DABCYL or its analogue, nanogold particle, black hole quencher, and not excluding others, the first and second oligonucleotides are the two primers of nucleic acid amplification reaction and are used such that the emission of the donor is quenched by the quencher/acceptor on the second oligonucleotide only in case of formation of primer dimer but in case of specific amplification product formation the above said quencher/acceptor of second oligonucleotide remains 10 –25 base pairs away and atleast 10 base pairs away from the donor moiety incorporated into the amplification product through the first oligonucleotide and at the same time the quencher of first oligonucleotides remains at least 10 bases away from the donor moiety incorporated into the amplification product through the first oligonucleotide and the 3' ends of the two labeled oligonucleotides remaining separated from each other by 10-25 base pairs in two opposite strands of amplification product thus allowing the donor moiety to emit its characteristic energy or light and signal generation for the detection or quantitation of a target nucleic acid sequence with increased signal to noise ratio.

18. A method as claimed in any of claims 1-17 wherein a first oligonucleotide labeled with a donor-1 moiety at or near preferably near its 3' end and a second oligonucleotide singly labeled at or near its 3' end with an acceptor moiety capable of absorbing the energy or light emitted by the donor-1 and a third oligonucleotide singly labeled with a donor-2 moiety at or near its 5' end and which is not extendable are provided; the donor-1 is capable of absorbing the energy or light emitted by the donor-2, the third oligonucleotide is fully or partly complementary to oligonucleotide-1 as long as the complementarity is maintained such that the donor-2 moiety remains quenched by the donor-1 moiety when the first oligonucleotide is not incorporated into the amplification product ; on incorporation of the first oligonucelotide and the second oligonucleotide into the amplification product the donor-2 moiety labeled third oligonucleotide gets separated from the donor-1 labeled first oligonucleotide and the emission of the donor-2 moiety is measured

19. A method as claimed in any one of claims 1-18 wherein said oligonucleotides are of the length 10 to 40 bases preferably 15 -30 bases and the said hair–pin oligonucleotides comprise anyone of the following:

a. a first oligonucleotide 10 – 40 bases long preferably 15 – 30 bases long fully complementary to the target nucleic acid sequence at the 5' end of which is attached a 5 – 9 bases long second oligonucleotide which may or may not be partially or fully complementary to the target sequence but fully complementary to the 3' end of the first oligonucleotide thus forming a stem and loop structure.

b. a first oligonucleotide of length between 15 – 40 preferably 15 – 30 bases fully complementary to the target nucleic acid sequence at the 5' end of which is attached a second oligonucleotide of length 2 to 12 bases and again at the 5' end of the second oligonucleotide is attached a third oligonucleotide of length 5 – 9 bases, the second and the third oligonucleotides may or may not be partly or fully complementary to the target nucleic acid sequence but the third oligonucleotide being fully complementary to 5 – 9 bases at or near the 3' end of the first oligonucleotide thus forming stem and loop structure.

c. a first oligonucleotide of length between 15 – 40 bases preferably between 15 – 30 bases fully complementary to the target nucleic acid sequence at the 5' end of the said first oligonucleotide is attached a second oligonucleotide of length 5 – 9 bases and at the 3' end of the said first oligonucleotide is attached a third oligonucleotide of length 5 – 9 bases, the second and the third oligonucleotides being fully complementary to each other, may or may not be fully or partly complementary to the target nucleic acid sequence thus forming a stem and a loop structure of said hair – pin oligonucleotide

d). a first oligonucleotide of length between 15 – 50 preferably 15 – 30 bases fully complementary to the target nucleic acid sequence at the 5' end of which is attached a second oligonucleotide of length 10 to 30 bases through a non-nucleotide organic linker the second oligonucleotide may

or may not be partly or fully complementary to the target nucleic acid sequence but the second oligonucleotide being fully or partly complementary to the bases at or near the 3' end of the first oligonucleotide.

20. A method as claimed in anyone of claims 1-19 wherein in case of quenched hair-pin, linear or otherwise oligonucleotide the nucleotide to which the donor or acceptor MET moiety is attached is either opposite or within 5 nucleotides away from the nucleotide to which the quencher for the respective donor or acceptor is attached, the donor/ acceptor MET moiety and the quencher being on two opposite strands.

21. A method as claimed in any one of claims 1 to 20 wherein said oligonucleotides are selected from DNA or RNA or chimeric mixtures or derivatives or modified versions thereof adapted for priming the amplification reaction or hybridizing to the amplified product and are deoxy oligonucleotides, oligonucleotide or peptide nucleic acid or modified oligonucleotides, the target nucleic acid sequence being selected from genomic DNA, m-RNA, RNA, c- DNA, chemically synthesized DNA or RNA,

22. A method as claimed in anyone of claims 1 to 21 wherein said oligonucleotides are amplification primers (forward and reverse) of polymerase chain reaction (PCR), reverse transcription PCR (RT-PCR), allele specific PCR, methylation status PCR, in – situ PCR, Triamplification, Nucleic acid sequence based amplification, immuno PCR and not excluding others.

23. A method as claimed in anyone of claims 1 to 22 used in real time RNA expression profiling by simultaneously quantitating large number of m-RNAs or C-DNAs using preferably PCR, RT – PCR, NASBA by using suitably labeled oligonucleotude primer pairs selected from individual m-RNAs or C-DNAs.

24. A method as claimed in anyone of claims 1 to 23 used in high throughput nucleic acid amplification reactions including PCR, RT-PCR and NASBA comprising providing first oligonucleotide amplification primer for each m-RNA or C-DNA in

large number from a pool selected from the sequences near the 5' ends of individual m-RNAs or C-DNAs and providing as second amplification primer a single common oligonucleotide primer (common for all m-RNAs or C-DNAs in the pool or sample) complementary to a sequence joined or ligated to 5' end of all m-RNAs or C-DNAs synthesized by reverse transcription in the pool or sample prior to the subjection to the amplification reaction, the oligonucleotide primers being said labeled oligonucleotides primer pairs either first oligonucleotide amplification primer is dual labeled quenched primer of the invention and the second common amplification primer is an unlabeled oligonucleotide or the first oligonucleotide amplification primer is labeled at or near preferably near 3' end with a donor or an acceptor MET moiety and the second common oligonucleotide amplification primer is labeled at or near preferably near 3' end with an acceptor or donor MET moiety respectively, which can also be provided quenched.

25. A method as claimed in any one of claims 1 to 23 used in high throughput nucleic acid amplification reactions including PCR, RT-PCR and NASBA comprising providing first oligonucleotide amplification primer for each m-RNA or C-DNA in large number from a pool selected from the sequences near the 3' or 5' ends of a restriction site of cDNAs of individual m-RNAs and providing as second amplification primer a single common oligonucleotide primer (common for all m-RNAs or C-DNAs in the pool or sample) complementary to a sequence joined or ligated to 3' and / or 5' ends of the restriction fragments of all C-DNAs synthesized by reverse transcription in the pool or sample prior to the subjection to the amplification reaction the oligonucleotide primers being said labeled oligonucleotides primer pairs, either first specific oligonucleotide amplification primer is dual labeled quenched primer of the invention and the second common amplification primer is an unlabeled oligonucleotide or the first oligonucleotide amplification primer is labeled at or near preferably near 3' end with a donor or an acceptor MET moiety and the second common oligonucleotide amplification primer is labeled at or near preferably near 3' end with an acceptor or donor MET moiety respectively, which can also be provided quenched.

26. A method as claimed in anyone of claims 1 to 24 used in RNA splice variant detection, wherein the target nucleotide sequence is a m-RNA or a C-DNA and the labeled oligonucleotides are either amplification primers (forward and reverse) of many nucleic acid amplification reactions including polymerase chain reaction (PCR), Reverse transcription polymerase chain reaction (RT-PCR), NASBA, one from 3' end of one exon and the other from 5' end of the adjacent exon or one of the two amplification primers of many nucleic acid amplification reactions including PCR, RT-PCR from 5' end of one exon and a probe or a third polymerase extendable oligonucleotide complemantary to 3'end of the adjacent exon.

27. A method as claimed in anyone of claims 1 to 25 wherein the detectable signal emitted by the acceptor MET moiety/ MET moieties is sizeable and more intense than the signal emitted by the same if there is no MET and the oligonucleotides are so designed that MET moieties come in right proximity such that MET between donor and acceptor moieties can occur and FRET is a preferred form of MET.

28. A method as claimed in anyone of claims 1 to 26 wherein the target nucleic acid sequence is an amplification product or the sequence of infectious disease agent for living organisms, or genomic sequence of a human, animal, plant or any other organism, mutation of which is implicated to the presence of a disorder or disease; or a human, animal or plant genomic sequence, the presence or absence of which is implicated to a disorder or disease; or a human, animal or plant genomic sequence, the presence or absence of which is implicated to susceptibility to an infectious agent; or a plant or any living organism genomic sequence, the presence or absence of which is implicated to a genetic trait or genotype of the plants, or the living organism; or a genomic sequence of an infectious agent, the presence or absence of which is implicated to strain typing.

29. A method as claimed in anyone of claims 4 to 27 wherein the donor and acceptor pair moieties are selected from any of the donor - acceptor MET \ FRET pairs known in the art and the donor moiety is preferably selected from the group consisting of fluorescein, carboxyfluoroscein (FAM), coumarin, 5-(2' amino ethyl) amino napthlein – 1- sulphonic acid(EDANS), rhodamine, anthranilamide, Reactive Red- 4,europium and

terbium chelate derivatives, a combination of an organic moiety having a large extinction coefficient of absorption and a fluorophore preferably of high quantum yield , and the said acceptor moiety is preferably selected from the group consisting of fluorescein, fluorescein derivatives like JOE and others, ethidium, texas red, eosin nitrotyrosine, malachite green, pyrene butyrate, Cy- 3 dye, Cy- 5 dye , DABCYL, DABCYL derivatives, rhodamine, rhodamine derivatives, nanogold particle black hole quencher and the quencher is selected from the group consisting of DABCYL and its derivatives, rhodamine, nanogold particles, black hole quencher and many other acceptor moieties.

30. A fast method as claimed in anyone of claims 4-7,12-13, 22-26, wherein the detection and/ or quantitation of amplified target nucleic acid is accomplished by amplifying a product incorporating the two amplification primers on two opposite strands separating their 3' ends by 0-25 nucleotide pairs,which improves the yield and specificity of the product and providing a double stranded DNA binding fluorescent dye selected from the group preferably consisting of ethidium bromide, CYBER TM GREEN I, pico green, acridine orange, thiazole orange Yo PRO-1 and chromomycin A3

31. A fast method as claimed in anyone of claims 4-7,12-13, 22-26, wherein the detection and /or quantitation of amplified target nucleic acid sequences is accomplished by providing the first oligonucleotide primer being labeled with a binding moiety preferably selected from biotin, magnetic particle , microsphere , a hapten or an anchor oligonucleotide attached directly or through a linker which can be respectively captured by streptavidin or magnet or centrifugation or anti-hapten antibody, capture oligonucleotide or the like and the second oligonucleotide primer being labeled with a signaling moiety like a fluorophore, rare earth metal chelate, biotin or a hapten, amplifying a product which incorporates the two amplification primers on two opposite strands separating their 3' ends by 0-25 nucleotide pairs,which improves the yield and specificity of the product and the signal is generated by inducing lumination, utilizing antihapten antibody-enzyme conjugate, (d) avidin/ steptavidin- enzyme conjugate and enzyme substrates, and other conjugates or by using unlabeled second oligonucleotide

primer and providing one or more fluorescently labeled nucleotide in appropriate concentration in the reaction mixture.

32. A method as claimed in anyone of claims 1 to 29 wherein the higher signal to noise ratio improvement is achieved by applying quenched labeled oligonucleotides (of hair-pin or otherwise configuration) of the invention and selecting an amplification product of the size, the size of the forward amplification primer plus the size of the reverse amplifcation primer plus 0-25 bases in the detection of a nucleic acid target sequence using MET/FRET between donor & acceptor moieties bringing the donor and the acceptor moieties close together.

33. A method as claimed in any one of claims 1-32 comprising multiplexing involving multiple pairs of MET labeled oligonucleotides for detection or quantification of multiple targets.

34. A method as claimed in anyone of claims 1 to 32 comprising heterogenous phase detection wherein one of the two amplification primers of many amplificaion reactions including PCR, RT-PCR, NASBA is fixed covalently through 5' end or an internal nucleotide to a solid support through a linker and spacer, and the other amplification primer is in aquaeous phase in contact with the solid phase, the said solid support to which the labeled oligonucleotide is attached is non – porous and transparent or translucent and glass or plastics like polystyrene, polyethylene, polypropylene or dextran and the like and preferably glass or glass wafer.

35. A method of claim 1-32 for high throughput hetrogeneous phase target nucleic acid detection wherein first of the two amplification primers of many target nucleic acids for many amplification reactions including PCR, RT-PCR, NASBA are fixed covalently through 5' end or an internal nucleotide to a solid support through a linker and spacer, and the second amplification primers are in aquaeous phase in contact with the solid phase, the said solid support to which the labeled oligonucleotide is attached is non – porous and transparent or translucent and glass or plastics like polystyrene, polyethylene, polypropylene or dextran and the like and preferably glass or glass wafer.

36. A method as claimed in any of claims 1-32 used in high throughput RNA expression profiling by many nucleic acid amplification reactions including PCR, RT-PCR and NASBA not excluding others by providing first oligonucleotide amplification primer for each m-RNAs or C-DNAs in large number from a pool selected from the sequences near the 5' ends of individual m-RNAs or C-DNAs, the said first oligonucleotide primers fixed covalently through 5' end or an internal nucleotide to a solid support through a linker and spacer, and the second amplification primers are in aquaeous phase in contact with the solid phase and providing as second amplification primer a single common oligonucleotide primer (common for all m-RNAs or C-DNAs in the pool or sample) complementary to a sequence joined or ligated to 5' end of all m-RNAs or C-DNAs in the pool or sample prior to the subjection to the amplification reaction the oligonucleotide primers being labeled oligonucleotides primer pairs of the invention, either first oligonucleotide amplification primer is dual labeled quenched primer of the invention and the second common amplification primer is an unlabeled oligonucleotide, or the first oligonucleotide amplification primer is unlabeled and the second common primer is a quenched or singly labeled unquenched oligonucleotide of the invention, or the first oligonucleotide amplification primer is labeled at or near preferably near 3' end with a donor or acceptor MET moiety and the second common oligonucleotide amplification primer is labeled at or near preferably near 3' end with an acceptor or donor MET moiety respectively and also can be provided quenched the solid support being non porous , transparent or translucent and glass plastics like polystyrene, polypropylene or dextran and the like and preferably glass or glass wafer.

37. A method as claim 1-32 used in high throughput RNA expression profiling by many nucleic acid amplification reactions including PCR, RT-PCR and NASBA not excluding others by providing first oligonucleotide amplification primer for each m-RNAs or C-DNAs in large number from a pool selected from the sequences near the 3' or 5' ends of a restriction site of individual m-RNAs or C-DNAs, the said first oligonucleotide primers fixed covalently through 5' end or an internal nucleotide to a solid support through a linker and spacer, and the second amplification primers are in aquaeous phase in contact with the solid phase and providing as second amplification

primer a single common oligonucleotide primer (common for all m-RNAs or C-DNAs in the pool or sample) complementary to a sequence joined or ligated to the 3' and 5' ends of all m-RNAs or C-DNAs restriction digested fragments in the pool or sample prior to the subjection to the amplification reaction, the oligonucleotide primers being labeled oligonucleotides primer pairs of the invention, either first oligonucleotide amplification primer is dual labeled quenched primer of the invention and the second common amplification primer is an unlabeled oligonucleotide or the first oligonucleotide amplification primer is labeled at or near preferably near 3' end with a donor or acceptor MET moiety and the second common oligonucleotide amplification primer is labeled at or near preferably near 3' end with an acceptor or donor MET moiety respectively and also can be provided quenched.

38. A fast method as claimed in anyone of claims 1-7, 22-26 and 27 wherein one or both the primers are provided labeled with a donor or an acceptor moiety and a double stranded DNA intercalating dye suitable to act as an acceptor or donor respectively at suitable concentration whereby on successful amplification donor/acceptor labeled primer/primers get incorporated into the amplification product bringing their 3' ends within 0-25 nucleotide pairs away and the double stranded DNA binding (intercalating) dye get intercalated into the amplification product thus bringing it close to the donor or acceptor moiety as the case may be and resulting in MET/FRET which can be measured, more specifically a fluoresecin labeled primer and double stranded DNA binding dye Ethidium bromide are used, where fluorescein is the donor and eithidium act as the acceptor for FRET to take place between the two.

39. A method of claims 1-36 used in closed tube format for detection or quantitation of one or more nucleic acid target sequences in real time.

40. A method as claimed in any one of claims 1 to 38 wherein the oligonucleotides used are selected from:

i) 5'-GGG GTA CTA CAG CGC CCT GA – 3'

ii) 5'-GGG GTA CTA CAG CGC CCT GA –3'

|

FAM

iii) 5'-GTC CTG GAA GAT GGC CAT GG –3'

iv) 5'-GTC CTG GAA GAT GGC CAT GG –3'

|

JOE

v) 5'-ATG GCC ATC GTC CTG GAA GAT GGC CAT GG –3'

vi) 5'-DABCYL-ATG GCC ATC GTC CTG GAA GAT GGC CAT GG -3'

|

JOE

vii) 5'-DABCYL-ATG GCC ATC GTC CTG GAA GAT GGC CAT GG –3'

|

FAM

viii) 5'-GCT CAT GGC GCC TGC CTG G –3'

|

DABCYL

ix) 5'-ATG GCC ATG TCC TGG AAG ATG GCC ATG G –3'

x). 5'– GGG GTA CTA CAG CGC CCT – 3'

|

FAM

xi).5'- GTC CTG GAA GAT GGC CAT GG –3'

|

Rhod

xii) 5'- GTC CTG GAA GAT GGC CAT GG – 3'

|

JOE

xii) 5' GGC AAT GAA AAG CCA CTT CT – 3' as a forward primer to amplify a 50 base pair segment (base position 23, 565-23,614) of E.coli genome.

xiii) 5' TTA ACC GGC GAT TGA GTA CC – 3' as a reverse primer to amplify a 50 base pair segment (base position 23,565-23,614) of E.coli genome.

41. A kit for use in method of analogous detection and / or quantitation of target nucleic acid sequence or sequences present in a sample comprising

- a polymerase or polymerases
- a first oligonucleotide of sequence complementary to the nucleotide sequence flanking a target nucleotide sequence suitably labeled with a donor MET/FRET moiety at or near 3' end, preferably near 3' end.
- a second oligonucleotide of sequence at 5' end of the first nucleotide sequence (d) flanking the target nucleotide sequence or the segment of the target nucleotide sequence suitably labeled with an acceptor MET/FRET moiety at or near 3' end, preferably near 3' end.
- deoxy nucleotides in solution (water or buffer) or lyophilized.
- a reaction buffer for the nucleic acid amplification reaction.

wherein the first and the second oligonucleotide sequences comprise at least the two primers (forward and reverse) of the nucleic acid amplification reactions and adapted to generate a detectable signal if the two oligonucleotides get incorporated into two opposite strands of amplified product bringing their 3' ends within 0-25 nucleotide pairs away and the donor and acceptor moieties come close together in right proximity.

42. A kit for use in method of analogous detection and/ or quantitation of target nucleic acid sequence or sequences present in a sample comprising:

a. A polymerase or polymerases.
b. A first oligonucleotide of nucleotide sequence complementary to the nucleotide sequence flanking a target nucleotide sequence suitably labeled with a donor MET/FRET moiety near 3' end.

c. A second oligonucleotide sequence at 5' end of the first nucleotide sequence flanking the target nucleotide sequence suitably labeled with an acceptor MET/FRET moiety near 3' end.

d. Deoxy nucleotides in solution (water or buffer) or lyophilized.

e. Reaction buffer for amplification reaction.

wherein the first and second oligonucleotide sequences are the two primers (forward and reverse) of nucleic acid amplification reaction; the first oligonucleotide sequence is suitably labeled at or near preferably near 3' end with a donor MET/FRET moiety; the second oligonucleotide sequence is labeled at or near preferably near 3' end with an acceptor MET/FRET moiety. Either the acceptor labeled second oligonucleotide or both the donor and acceptor label oligonucleotides are provided quenched using one or two additional oligonucleotides as such or suitably labeled with quencher in a hair-pin configuration of the oligonucleotides or linked through non-nucleotide organic linker or otherwise as mentioned in claims 11 and 20 and adapted to generate a detectable signal if the two oligonucleotide primers get incorporated into two opposite strands of amplified product bringing their 3' ends within 0-25 nucleotide pairs away and the donor and acceptor moieties come close together in right proximity.

43. The kit as claimed in anyone of claims 41 to 42 wherein additionally positive control template and suitable MET/FRET labeled primers are also included as control for amplification reaction.

44. A Kit as claimed in claim 41 for use in method of claims 12 to 16 of analogous detection and/or quantitaiton of target nucleic acid sequence or sequences present in a sample comprising

a. polymerase or polymerases.

b. a first oligonucleotide of sequence complementary to the nucleotide sequence flanking a target nucleotide sequence or a segment of a target nucleotide sequence and a second oligonucleotide of sequence at the 5' end of the first oligonucleotide sequence (d) flanking the target nucleotide sequence or the segment of target nucleotide sequence.

c. a third oligonucleotide of sequence complementary to the target nucleotide sequence or the segment of the target nucleotide sequence.

d. deoxynucleotides in solution (water or buffer) or lyophilized.

e. reaction buffer for nucleic acid amplification reaction.

f.

g. wherein the first and second oligonucleotide sequences are the two primers (forward and reverse) of nucleic acid amplification reaction and the third oligonucleotide sequence is either polymerase extendable or non extendable probe. The first oligonucleotide is suitably labeled with a first moiety of a MET/FRET pair at or near 3' end and the third oligonucleotide is labeled at or near 3'end with the second moiety of the MET/FRET pair, and a detectable signal is generated when the first oligonucleotide gets incorporated into one of the two strands of the amplification product and the third oligonucleotide either hybridize to the strand of the amplification product into which MET/FRET labeled primer gets incorporated into or get incorporated into the opposite strand if it is a primer (nested) thus bringing the 3' ends of the first and the third oligonucleotides in two opposite strands within 0-25 nucleotide pairs away and the donor acceptor moieties come close together in right proximity.

45. A kit as claimed in claim45 for use in method of claims 12 to 16 of analogous detection of target nucleic acid sequence or sequences present in a sample comprising.

a. a polymerase or polymerases.

b. a first oligonucleotide of sequence complementary to the nucleotide sequence flanking a target nucleotide sequence or a segment of target nucleotide sequence and a second oligonucleotide sequence at the 5' end of the first oligonucleotide sequence (d) flanking the target nucleotide sequence or the segment of the target nucleotide sequence.

c. a third oligonucleotide of sequence complementary to the target nucleotide sequence or the segment of the target nucleotide sequence amplified by amplification reaction.
d. deoxynucleotides in solution (water or buffer) or lyophilized.
e. reaction buffer for nucleic acid amplification reaction

wherein the first and second oligonucleotide sequences are the two primers (forward and reverse) of nucleic acid amplification reaction; the first oligonucleotide sequence is suitably labeled at or near 3' end with a donor MET/FRET moiety; the third oligonucleotides sequence is labeled at or near 3' end with an acceptor MET/FRET moiety and carries at its 5' end a fourth oligonucleotide sequence 5-9 nucleotide long fully complementary to a part of third oligonucleotide sequence so that the third oligonucleotide forms a hair – pin 5 to 9 nucleotide pair stem and loop structure with the fourth oligonucleotide and the fourth oligonucleotide additionally carries a quencher at or near its 5' end, the third and fourth oligonucleotides can also be separate and joined together thorough small oligonucleotide or a linker as mentioned in claims 11 and 19 such that the MET/FRET moiety on the third oligonucleotide can remain quenched when the third oligonucleotide is not hybridized to or not incorporated into the amplification product.

46. A kit as claimed in claim 41 and 42 wherein at least the first oligonucleotide is provided labeled near 3' end with a donor MET/FRET moiety and a double stranded DNA intercalating dye capable of absorbing energy or light emitted by the donor moiety and emitting energy or light is also provided.

47. A kit as claimed in claim anyone of claims 41 to 42 wherein at least the first oligonucleotide is labeled near 3' end with an acceptor MET/FRET moiety and a double stranded DNA intercalating dye (donor) capable of emitting energy or light on illumination is provided such that the acceptor moiety is capable of absorbing the energy or light emitted by the intercalating dye and emitting energy or light.

48. A kit as claimed in anyone of claims 41 to 45 comprising multiple oligonucleotide sets of claim for detection and/or/quantitation of multiple target sequences.

49. A kit for the detection of target nucleic acid sequences or sequences using the oligonucleotides used in the methods of claims 4 to 7 and 31 wherein the detection and /or quantitation of amplified target nucleic acid sequences is accomplished by providing the first oligonucleotide primer being labeled at or near 5'end with a binding moiety preferably biotin, or magnetic particle or microsphere or a hapten or the like or attached to an anchor oligonucleotide directly or through a linker which can be respectively captured by streptavidin or magnet or centrifugation or anti-hapten antibody, capture oligonucleotide or the like and the second oligonucleotide primer being labeled with a signaling moiety like a fluorophore, rare earth metal chelate, biotin or a hapten, amplifying a product which incorporates the two amplification primers on two opposite strands separating their 3' ends by 0-25 nucleotide pairs and the signal is generated by inducing lumination, the hapten being detected utilizing anti-hapten antibody-enzyme conjugate, biotin being detected by avidin/ steptavidin- enzyme conjugate and enzyme substrates, and other conjugates or by using unlabeled second oligonucleotide primer and providing atleast one fluorescently labeled nucleotide in the reaction mixture in appropriate concentration.

50. A kit or kits for the detection of target nucleic acid sequences providing all or more components and using the oligonucleotides for detection used in methods of claims 4 to 7 and 31 unlabeled wherein the detection and/ or quantitation of amplified target nucleic acid is accomplished by providing double stranded DNA binding fluorescent dye selected from the group consisting of eithidium bromide, CYBER TM GREEN I, pico green, acridine orange, thiazole orange Yo PRO- 1 and chromomycin A3 but not excluding others.

51. The kit as claimed in any one of claims 41 to 50 wherein the oligonucleotides used are selected from:

i) 5'-GGG GTA CTA CAG CGC CCT GA – 3'

ii) 5'-GGG GTA CTA CAG CGC CCT GA –3'

|

FAM

iii) 5'-GTC CTG GAA GAT GGC CAT GG –3'

iv) 5'-GTC CTG GAA GAT GGC CAT GG –3'

|

JOE

v) 5'-ATG GCC ATC GTC CTG GAA GAT GGC CAT GG –3'

vi) 5'-DABCYL-ATG GCC ATC GTC CTG GAA GAT GGC CAT GG -3'

|

JOE

vii) 5'-DABCYL-ATG GCC ATC GTC CTG GAA GAT GGC CAT GG –3'

|

FAM

viii) 5'-GCT CAT GGC GCC TGC CTG G –3'

|

DABCYL

ix) 5'-ATG GCC ATG TCC TGG AAG ATG GCC ATG G –3'

x). 5'– GGG GTA CTA CAG CGC CCT – 3'

|

FAM

xi).5'- GTC CTG GAA GAT GGC CAT GG –3'

|

Rhod

xii) 5'- GTC CTG GAA GAT GGC CAT GG – 3'

|

JOE

xii) 5' GGC AAT GAA AAG CCA CTT CT – 3' as a forward primer to amplify a 50 base pair segment (base position 23, 565-23,614) of E.coli genome.

xiii) 5' TTA ACC GGC GAT TGA GTA CC – 3' as a reverse primer to amplify a 50 base pair segment (base position 23,565-23,614) of E.coli genome.

52. A method of manufacture of a kit for use in method of analogous detection and / or quantitation of target nucleic acid sequence or sequences present in a sample comprising

(a) providing a polymerase or polymerases.

(b) providing a first oligonucleotide of sequence complementary to the nucleotide sequence flanking a target nucleotide sequence suitably labeled with a donor MET/FRET moiety at or near 3' end.

(c) providing a second oligonucleotide of sequence at 5' end of the first nucleotide sequence (d) flanking the target nucleotide sequence or the segment of the target nucleotide sequence suitably labeled with an acceptor MET/FRET moiety at or near 3' end.

(d) providing deoxy nucleotides in solution (water or buffer) or lyophilized.

(e) providing a reaction buffer for the nucleic acid amplification reaction.

wherein the first and the second oligonucleotide sequences comprise the two primers (forward and reverse) of many nucleic acid amplification reactions and adapted to generate a detectable signal if the two oligonucleotides get incorporated into two opposite strands of the amplified product with their 3' ends remaining seperated from each other by 0-25 nucleotide pairs and the donor and the acceptor moieties come close together in right proximity and the first and second oligonucleotides are any of the labeled unquenched or quenched oligonucleotide primers of the claims 1-28.

53. A method for high throughput RNA expression profiling for analysis of absolute quantaties of mRNAs carried out in homogenous or heterogeneous phase using the

method of detection/quantification of target nucleic acid sequence as claimed in anyone of claims 1 to 37 and a kit for the same including all requisite components.

54. The method as claimed in anyone claims 1-22 for the heterogeneous mutation detection comprising two amplification primer oligonucleotides of the invention one being labeled with a donor MET momety near 3' end and the other being labeled with an acceptor MET moiety near 3' end, carrying out target amplification reaction and thermal denaturation analysis of the amplification product or products thus amplified, the same method where the labeled oligonucleotides are also quenched oligonucleotides of the invention and a kit for the same.

55. The method as claimed in anyone of preceding claims used in high throughout nucleic acid amplification reactions including PCR, RT-PCR and not excluding others.

56. The method as claimed in anyone of preceding claims wherein the donor moiety is a combination of an organic moiety having a large extinction coefficient of absorption and a fluorophore preferably of high quantum yield and extinction coefficient .

57. The method as claimed in anyone of preceding claims wherein the higher signal to noise ratio is achieved by applying hair-pin or otherwisequenched labeled oligonucleotides of the invention in ligase chain reaction.

58. A method and a kit as claimed in anyone of preceding claims wherein the higher signal to noise ratio improvement is achieved by applying hair-pin quenched or otherwise quenched labeled oligonucleotides of the invention in the detection of a nucleic acid target sequence using MET/FRET between donor and acceptor moieties on two oligonucleotides (probes) designed against one strand of the target sequence in combination with two amplification primers which are unlabeled or on one oligonucleotide primer and one oligonucleotide probe.

59. The method and a kit as claimed in claims 4--12 of preceding claims wherein one or both the amplification primers are labeled at or near 3' end preferably near 3' end

with acceptor or donor moiety or moieties and atleast one of the four deoxynucleotides is provided labeled with the donor or acceptor moiety respectively in appropriate concentrations and composition, and wherein on incorporation of the acceptor or donor labeled primer or primers and the respective donor or acceptor labeled nucleotide into the amplification product which brings the 3' ends of the two primers in two opposite strands seperated from each other by 0-25 nucleotide pairs and a signal is generated by MET/FRET between the donor and the acceptor moieties .

60. The kit as claimed in anyone of preceding claims wherein the polymerases are a reverse transcriptase, T7RNA polymerase and a DNA polymerase,.

61. A method of detection of target nucleic acid sequence , a kit used for the same and its process of manufacture substantially as herein described and illustrated with reference to examples and figures and many modifications thereof .

STATEMENT UNDER ARTICLE 19 (1)

The invention relates to method and kit for detection and or quantification of target nucleic acid sequences based on the amplification of a segment of a target nucleic acid utilizing two amplification primers (forward and reverse) which are labeled separately near 3' ends with donor or acceptor MET/FRET moiety so configured that the amplification product of the size of forward primer plus reverse primer plus 0-25 bases is formed. On amplification oligonuleotides are incorporated in the opposite strands of amplification product with their 3'ends at a distance of 0-25 nucleotide pairs and their 5' ends remaining further away. This brings the signaling MET/FRET moieties on two primers, or one primer and a probe, which otherwise remain separated in absence of amplification, together and signal is generated by energy transfer where donor is excited and acceptor and/or donor emission is measured.

U.S. 5,866,336 teaches labeled primer and labeled blocking oligonucleotide used in hair-pin quenched configuration or otherwise for detection of target nucleotide sequence and amplification and a method for the same and a kit. In the process a signal is generated due to separation of the donor and the acceptor moieties, which otherwise remain close together in absence of amplification, whether polymerase chain reaction or triamplification is used for target amplification. The donor and the quencher (acceptor) moieties are on the same oligonucleotide primer or on one primer and the blocking oligonucleotide, which is complementary to the said primer. The signal is generated on disruption of energy transfer and there is no interaction between the FRET moieties on two separate primers.

US 6,287,781 uses a MET/FRET labeled probe for hybridization to one of the strands of the amplification product into which a fluorescent labeled nucleotide is incorporated along with other nucleotides. On successful amplification of target nucleotide the labeled nucleotide get incorporated into the amplification product and on hybridization of the labeled probe to the amplified product there is FRET or energy transfer between the moieties on the probe and the labeled nucleotide.

None of U.S. 5,866,336 and US 6,287,781 teaches use of two labeled primers with specific distance of 0-25 nucleotides at which the 3'ends of donor and acceptor labeled primers are brought in the opposite strands upon amplification in amplification product, and signal generation due to energy transfer between them on opposite strands without any further manipulation.. Further in U.S. 5,866,336 either the two moieties are present together on the same oligonucleotide or, in case of triamplification are on one primer and a blocking oligonucleotide, which is complementary to the labeled primer and signal is generated after further manipulation of disrupting the energy transfer between the donor and acceptor moieties on separation after the amplification. While US 6,287,781 uses probes for hybridization in combination with a labeled nucleotide. In contrast in the present invention the labeled oligonucleotides are either both primers or one primer and one probe but not probe in combination with a labeled nucleotide

To emphasize the above distinguishing features the applicant wishes to amend the claims under Article 19 (PCT) based on the description in the text and exemplary illustrations.